The Plant Cell, Vol. 6, 1135–1143, August 1994

Analysis of a Soluble Calmodulin Binding Protein from Fava Bean Roots: Identification of Glutamate Decarboxylase as a Calmodulin-Activated Enzyme

Vincent Ling,[a,1] Wayne A. Snedden,[b] Barry J. Shelp,[b] and Sarah M. Assmann [a,2]

[a] Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138
[b] Department of Horticultural Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The identity of a soluble 62-kD Ca^{2+}-dependent calmodulin binding protein (CaM-BP) from fava bean seedlings was determined. Using ^{125}I-CaM overlay assays, a class of soluble CaM-BPs was detected in extracts of tissues comprising the axis of 1.5-week-old seedlings, excluding the root tip and emergent leaves. The size of these CaM-BPs was not uniform within all parts of the plant; the apparent molecular masses were 62 kD in roots, 60 kD in stems, and 64 kD in nodules. The root 62-kD CaM-BP was purified, and internal microsequence analysis was performed on the protein. A tryptic peptide derived from the CaM-BP consisted of a 13-residue sequence corresponding to a highly conserved region of glutamate decarboxylase (GAD), an enzyme that catalyzes the α-decarboxylation of glutamate to form the stress-related metabolite γ-aminobutyrate. Activity assays of partially purified, desalted, root GAD revealed a 50% stimulation by the addition of 100 μM Ca^{2+}, a 100% stimulation by the addition of 100 μM Ca^{2+} plus 100 nM CaM, and no appreciable stimulation by CaM in the absence of added Ca^{2+}. The demonstration that plant GAD is a Ca^{2+}-CaM-stimulated enzyme provides a model in which stress-linked metabolism is modulated by a Ca^{2+}-mediated signal transduction pathway.

INTRODUCTION

Calmodulin (CaM) is a Ca^{2+} binding protein involved in numerous Ca^{2+}-dependent signaling pathways in eukaryotic cells (Roberts and Harmon, 1992). In plants, CaM is implicated in many Ca^{2+}-dependent responses, including phytochrome action (Neuhaus et al., 1993), gravitropism (Stinemetz et al., 1987), and thigmomorphogenesis (Braam and Davis, 1990). CaM concentrations vary between different plant tissues, and some CaM binding proteins (CaM-BPs) show tissue and cell specificity (Ling and Assmann, 1992). In plant cells, transient influxes of Ca^{2+} show different kinetics depending on the type of stimulus applied (Knight et al., 1991). Because physiological responses vary among tissues that may utilize CaM as a common second messenger (Roberts and Harmon, 1992), these data suggest that multiple CaM-dependent pathways arise during plant development.

Extremely little is known about the biochemical pathways modulated by CaM. To date, only four CaM-stimulated enzymes from plants have been well characterized: NAD kinase, Ca^{2+}-ATPase, nuclear nucleoside triphosphatase (Roberts and Harmon, 1992), and a slow vacuolar ion channel (Bethke and Jones, 1994). However, by using ^{125}I-CaM overlay assays, more than a dozen CaM-BPs of various molecular masses have been detected within different organs, tissues, and protoplast types of fava bean plants (Ling and Assmann, 1992). One set of prominent Ca^{2+}-dependent CaM-BPs in the range of 60 to 64 kD was found in stem and root tissue (Ling and Assmann, 1992). Here, we present data concerning the spatial distribution of this set of soluble CaM-BPs and identification of the soluble root protein as glutamate decarboxylase (GAD). Recently, a petunia GAD cDNA clone was isolated by screening a cDNA library using radiolabeled CaM as a ligand probe (Baum et al., 1993). However, the authors did not succeed in demonstrating regulation of petunia GAD activity by CaM, and concluded with a suggestion that CaM association with GAD may function to stabilize and compartmentalize GAD within the cell. In distinct contrast, we provide direct evidence that fava bean root GAD is a bona fide Ca^{2+}-dependent CaM-stimulated enzyme, thus providing a link between Ca^{2+} signaling and γ-aminobutyrate (GABA) synthesis.

Although GAD activity and its product GABA have been known in legumes for decades (Kulkarni and Sohonie, 1956), the functional role of GABA in plants is not well established. As a widely distributed and highly accumulated non-protein plant amino acid, GABA has been hypothesized to be part of a metabolic mechanism for the control of pH, an amino acid metabolite, a nitrogen storage compound, and/or an insect

[1] Current address: Genetics Institute, 87 CambridgePark Drive, Cambridge, MA 02140.
[2] To whom correspondence should be addressed at: The Pennsylvania State University, Department of Biology, 208 Mueller Laboratory, University Park, PA 16802.

neural inhibitor synthesized during plant stress (Bown and Shelp, 1989; Satya Narayan and Nair, 1990). Because both Ca^{2+} and GABA levels increase rapidly during some stress responses (Wallace et al., 1984; Knight et al., 1991), Ca^{2+}-CaM modulation of GAD may serve in vivo as a regulatory mechanism for environmental induction of GABA accumulation.

RESULTS

CaM-BPs along the Axis

The distribution of CaM-BPs in protein extracts from axially dissected fava bean seedlings was determined by using ^{125}I-CaM overlay assays. In a 21-cm seedling, an axially localized CaM-BP of ~62 kD was detected in the regions from 2 to 17 cm behind the root tip, including the root–shoot transition zone (Figure 1A). Although a single 62-kD band was usually detected, the band could sometimes be resolved as a tightly migrating doublet, suggesting the presence of protein isoforms. The 62-kD protein was never detected in the first centimeters of root tips in either primary (Figure 1) or axillary (data not shown) roots. In a 32-cm seedling with an 8-cm stem and clearly discernible leaves, the CaM-BP was also detected in the stem region above the root–shoot transition zone (Figure 1B). Three centimeters past the transition zone and into the stem region, however, the molecular mass of the CaM-BP decreased slightly but reproducibly to 60 kD. This difference in molecular mass was clearly visible in overlays of extracts derived from whole organs (Figure 1C). The decrease in molecular mass may be a result of protein isoforms or secondary modifications and may account for the size difference in CaM-BPs between seedling roots (62 kD) and stems of mature plants (60 kD) described previously (Ling and Assmann, 1992). The CaM binding signal from the 60-kD protein greatly diminished in intensity further up the stem toward the area of the emergent leaf. This observation is consistent with previous ^{125}I-CaM overlays that indicated the absence of a CaM-BP in the 62-kD range in mature leaf extracts (Ling and Assmann, 1992).

CaM-BPs within Component Root Tissues

To determine the location of the 62-kD protein in root tissues, the cortex and stele were dissected from 1.5-week-old fava bean roots (Leonard et al., 1975). Protein extracts made from each tissue were centrifuged at 150,000*g* for 1 hr to separate soluble proteins from proteins cosedimenting with the microsomal pellet. The ^{125}I-CaM overlay assay demonstrated that the 62-kD protein was the predominant CaM-BP in the supernatant fractions of both cortex and stele (Figure 2). Corresponding assays of stem tissue revealed the stem 60-kD CaM-BP to be the predominant soluble CaM-BP of those samples (data not shown). The slightly lower levels of the 62-kD CaM-BP detected in supernatant samples compared to corresponding total protein

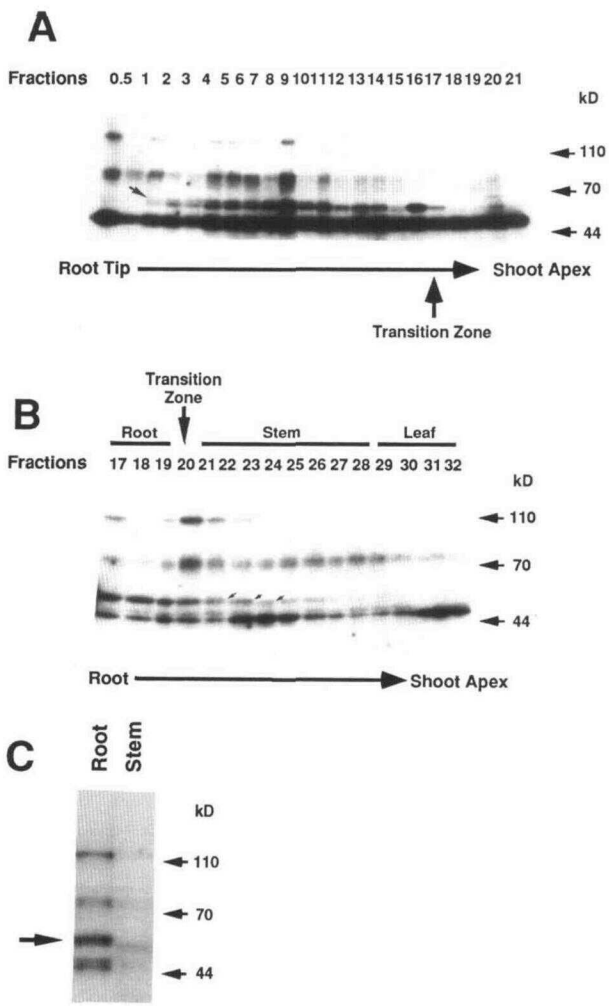

Figure 1. ^{125}I-CaM Overlay Assay of Protein Extracts Derived from Axially Sectioned Fava Bean Seedlings.

(A) Protein extracts (20 μg) from sections starting from root tip to shoot apex in a 21-cm seedling. Fraction numbers denote the section taken as measured from the root tip; the first two fractions indicate samples taken from the first two 0.5 cm of the root tip, and fractions 2 to 21 denote 1-cm segments for the rest of the axis. The root–shoot transition zone is indicated. Numbers at right indicate apparent molecular masses in kilodaltons. The arrow on the blot denotes the location of the 62-kD CaM-BP.

(B) Protein extracts (20 μg) from sections of a plumule from a 32-cm seedling. Each fraction represents a 1-cm section of the axis, from 17 cm above the root tip to the shoot apex containing 4 cm of developing leaf. The arrows on the blot denote the observed mobility shift of the CaM-BP. Numbers at right indicate apparent molecular masses in kilodaltons.

(C) Protein extracts (40 μg) of 1.5-week-old seedling organs. Total root and total stem extracts are displayed. The arrow at left denotes the position of the root 62-kD CaM-BP. Numbers at right indicate apparent molecular masses in kilodaltons.

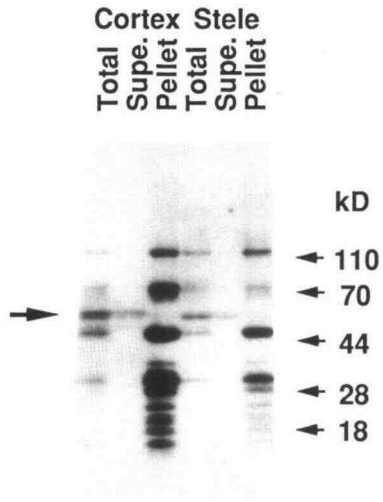

Figure 2. ^{125}I-CaM Overlay Assay of Protein Extracts from Root Tissues of Fava Bean Seedlings.

Protein extracts (20 μg) from a dissected cortex and stele were analyzed. Total, total proteins; Supe., proteins in the supernatant fraction following 150,000*g* (1 hr) centrifugation; Pellet, proteins in the microsomal membrane pellet following 150,000*g* (1 hr) centrifugation. The arrow at left indicates the position of the soluble CaM-BP. Numbers at right indicate apparent molecular masses in kilodaltons.

samples may have been the result of protein loss or proteolysis during manipulations involved with protein separation; the 62-kD protein was found to be highly labile during the initial protein extraction steps. It is interesting to note that most CaM-BPs in both cortex and stele cosedimented with the microsomal pellet and that the CaM-BP profiles for these two tissues were virtually identical. Distinct classes of CaM-BPs previously mentioned (Ling and Assmann, 1992) were clearly discernible: the ubiquitously distributed CaM-BPs (52, 78, and 115 kD) and the smaller CaM-BPs (molecular masses of less than 30 kD) were associated with the microsomal pellet, and the 62-kD CaM-BP was associated with the soluble fraction. Studies of CaM-BPs associated with carrot membrane preparations showed analogous CaM-BPs of ~115, 78, and 52 kD that partition between 22 to 32% sucrose, a fraction that is enriched for plasma membrane proteins (D.M. Ratterman, V. Ling, S.M. Assmann, and H. Sze, unpublished data).

Nodule CaM-BPs

In legumes, control of nodule development and function is coordinated between the plant and nitrogen-fixing bacteria at the physiological and molecular level (Fischer and Long, 1992). To investigate whether CaM-BPs might be involved in this process, mature fava bean roots and nodules were harvested 5 to 6 weeks after inoculation with *Rhizobium leguminosarum* and examined by ^{125}I-CaM overlay assays. A very strong 64-kD CaM-BP signal was detected in nodule samples (Figure 3A). Upon ultracentrifugation, the 64-kD CaM-BP signal partitioned to the soluble fraction (Figure 3B). Similar to the results in roots, the intensity of the 64-kD CaM-BP signal was lower in the soluble fraction than in the crude extract. No CaM-BPs in this size range were detected in *R. leguminosarum* extracts.

Protein Purification

The 62-kD CaM-BP from the root was partially purified to identify the protein. Although the soluble CaM-BP was amenable to purification steps involving ammonium sulfate precipitation and DEAE-cellulose, reactive blue 2, and CaM-agarose chromatography, the yield was optimized by using only DEAE-cellulose anion exchange chromatography and CaM-agarose affinity chromatography. Purification data indicated that the 62-kD CaM-BP comprised less than 0.5% of total protein mass extracted from mature fava bean roots (Table 1).

From 2.4 kg of roots, a sufficient quantity of the 62-kD CaM-BP was isolated and accumulated for tryptic digestion and internal sequence determination. Of 14 residues

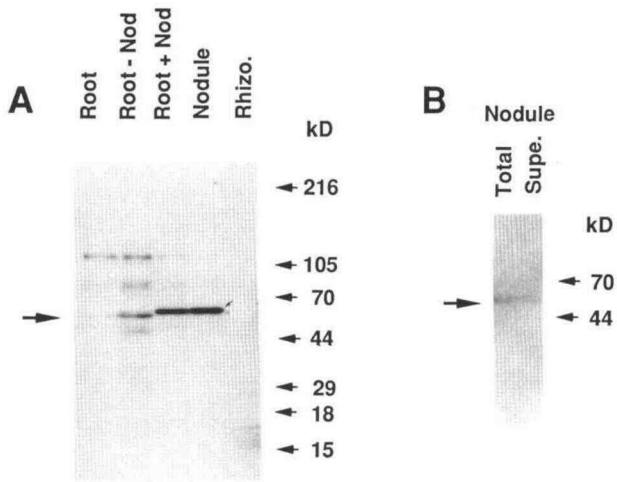

Figure 3. ^{125}I-CaM Overlay Assay of Nodule Proteins.

(A) Analysis of protein extracts (40 μg) from mature root and nodule samples. Root, uninoculated root; Root – Nod, 3-cm section of nodule-bearing root with nodules removed; Root + Nod, 3-cm section of nodule-bearing root; Nodule, nodule extract, Rhizo., cultured *R. leguminosarum* cells. The arrow on the blot indicates the position of 64-kD CaM-BP in nodules. The arrow at left indicates the position of the root 62-kD CaM-BP. Numbers at right indicate apparent molecular masses in kilodaltons.

(B) Analysis of soluble proteins extracted from fava bean nodules by centrifugation at 150,000*g* (1 hr). Total, total protein extract (40 μg); Supe., soluble proteins from supernatant fraction (40 μg). The arrow at left indicates the position of the CaM-BP. Numbers at right indicate apparent molecular masses in kilodaltons.

Table 1. Purification of the 62-kD CaM-BP from Fava Bean Root (400 g)

Fraction	mg/mL	mg Protein[a]	Binding Activity[b]	Sp. Activity[c]	Purification	Yield (%)
Crude	1.3	754	4109	5.45	1	100
Supernatant	1.6	864	4493	5.20	0.9	109
DEAE-cellulose	2.8	98	936	9.55	1.8	23
CaM-agarose	0.15	0.36	56	155.5	28.5	1.4
Excised band	NA[d]	1.4×10^{-3}	56	40000	7340	1.4

[a] Protein concentration was determined with the Bio-Rad protein assay kit, except for the excised band sample; for this sample, protein concentration was determined by mass spectrometry analysis.
[b] Binding activity indicates ^{125}I-CaM binding capacity based on densitometric scanning of autoradiographs from the overlay assay performed in parallel with all samples described.
[c] Specific activity is based on binding activity per milligram of protein in the fraction.
[d] NA, not applicable.

sequenced and 13 amino acids identified, significant sequence homologies were found with GADs from petunia, *Escherichia coli*, Drosophila, and cat (Figure 4). The derived amino acid sequence of petunia GAD (Baum et al., 1993) has the highest degree of sequence identity with the fava bean peptide fragment, with 100% predicted identity over the amino acids determined (residues 234 to 246). The peptide corresponds to a sequence in petunia GAD directly adjacent to a lysine residue at position 233, consistent with this amino acid serving as a predicted site of trypsin cleavage in proteins. In petunia GAD, this region is 26 amino acid residues upstream from the pyridoxal phosphate cofactor binding site of GAD, an active site possessing the highest degree of sequence conservation between prokaryotic and eukaryotic pyridoxal phosphate–dependent decarboxylases (Jackson, 1990; Maras et al., 1992). In agreement with our work, a CaM binding domain was localized to the C terminus of a recombinant petunia GAD (Baum et al., 1993).

GAD Activity Assays

GAD activity was assayed by scintillation spectroscopy of L-1-^{14}C-glutamate–dependent $^{14}CO_2$ production (Snedden et al., 1992). Root GAD was partially purified for this assay by extraction in EDTA, ammonium sulfate precipitation, membrane filtration, and HPLC DEAE-anion exchange. In this study, the Ca^{2+}-independent activity of GAD could not be measured by assaying in the presence of EDTA or EGTA because these chelators were found to competitively inhibit GAD activity (W.A. Snedden and B.J. Shelp, unpublished data). The extent to which unstimulated GAD activity was Ca^{2+}- and CaM-independent in our assay procedure could not be assessed because the removal of Ca^{2+}-CaM intrinsically bound to GAD during purification was not monitored. Root GAD activity was stimulated ~50% by the addition of 100 μM Ca^{2+} and ~100% by the addition of 100 μM Ca^{2+} plus 100 nM CaM (Table 2). No significant stimulation of GAD was seen by the addition

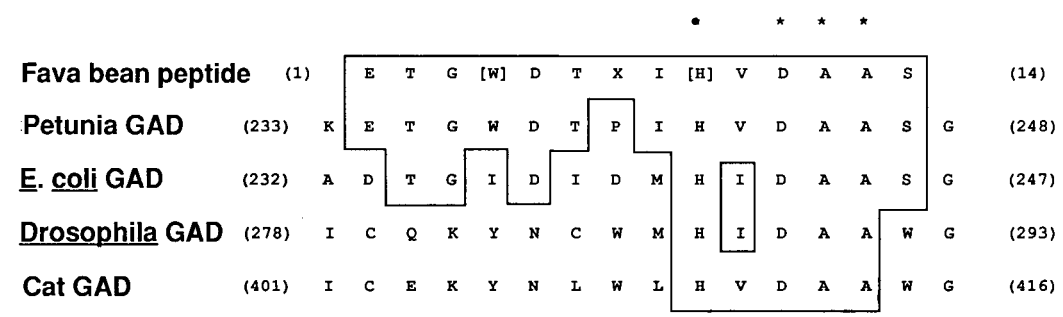

Figure 4. Amino Acid Sequence of the Internal Peptide from the Root 62-kD CaM-BP and Comparison with Glutamate Decarboxylases from Other Sources.

CaM affinity-purified protein fractions were separated by SDS-PAGE, followed by electrotransfer onto PVDF membranes and Ponceau S staining. The 62-kD CaM-BP was excised, eluted from the PVDF membrane, and trypsin digested; the resultant peptides were separated by HPLC. The amino acid sequence (one-letter notation) determined from one peptide is presented. X indicates the position in the sequence where the residue could not be determined, and bracketed residues indicate ambiguity associated with that amino acid determination. Numbers in parentheses indicate residue number in the sequence. The fava bean peptide is aligned with GADs from petunia (Baum et al., 1993), *E. coli* (Maras et al., 1992), Drosophila (Jackson, 1990), and cat (Kobayashi et al., 1987). Sequence identity is displayed. Asterisks indicate conserved residues between GADs of different species (Jackson, 1990).

Table 2. Effect of Ca^{2+} and CaM on Fava Bean Root GAD Activity[a]

Treatment [b]	Activity: nmol $^{14}CO_2$/min/mg Protein	% Control
$-Ca^{2+}$, −CaM (control)	16.0 ± 0.59	100
$+Ca^{2+}$ (100 μM)	24.6 ± 0.52	154
+CaM (100 nM)	18.2 ± 0.73	114
$+Ca^{2+}$ (100 μM), +CaM (100 nM)	34.0 ± 0.18	212

[a] Assays were performed as described in Methods. Data represent the mean of three replicates ± SE from an extract of 15 fava bean seedling roots. GAD activity was not detected in controls lacking root extract (data not shown).

[b] $+Ca^{2+}$ and +CaM indicate the addition of Ca^{2+} and CaM, respectively, to the assay. $-Ca^{2+}$ and −CaM indicate that no exogenous Ca^{2+} and CaM was added to assay.

of 100 nM CaM in the absence of added Ca^{2+}, demonstrating GAD to be a Ca^{2+}-CaM–stimulated enzyme. During subsequent GAD assay development, preliminary experiments performed in the presence of the protease inhibitors leupeptin, pepstatin, and antipain frequently resulted in up to 500% stimulation of GAD by Ca^{2+} plus CaM (W.A. Snedden and B.J. Shelp, unpublished data). Thus, the data presented in Table 2 most probably represent a lower estimate of the degree of GAD activation by Ca^{2+} plus CaM, and further optimization of the GAD assay may reveal higher levels of stimulation. These results are distinct from those of Baum et al. (1993); they reported the complete absence of stimulation of petunia GAD in vitro by Ca^{2+}-CaM, based on GAD assays using thin-layer chromatography to identify newly synthesized GABA. The discrepancy between these reports may be a result of differences in the thoroughness of endogenous CaM removal during protein purification and/or the sensitivity of the type of GAD assay used. Unless precautions are taken to remove protein and membrane-bound CaM, residual CaM present in final protein preparations is known to result in high background activity of CaM-activated enzymes (Rasi-Coldogno et al., 1993).

DISCUSSION

The identification of the soluble 62-kD CaM-BP in roots as GAD is consistent with the pattern of GABA accumulation; GABA is found in high concentrations in phloem sap, xylem sap, and root nodules (see reviews by Bown and Shelp, 1989; Satya Narayan and Nair, 1990). In cotton plants, lack of root aeration increases GABA accumulation in roots and xylem by four- to fivefold, whereas no increase of GABA levels in leaves is detected (Guinn and Brinberhoff, 1970). In addition, significant increase in GABA concentration occurs in intact tissues minutes after stimulation by mechanical bending, tissue damage, cold shock, and light–dark transition (Wallace et al., 1984). These same stimuli elicit Ca^{2+} and/or CaM responses (Braam and Davis, 1990; Knight et al., 1991). A role for Ca^{2+} in the potentiation of GAD activity had been postulated (Wallace et al., 1984), although no biochemical linkage of GAD to Ca^{2+} or CaM action was known at that time. We demonstrated that fava bean root GAD is a Ca^{2+}-dependent CaM-BP by affinity chromatography and ^{125}I-CaM binding (Table 1; Ling and Assmann, 1992) and have made the novel observation that the activity of GAD is stimulated twofold over the control by Ca^{2+}-CaM in in vitro assays (Table 2). This degree of stimulation of fava bean GAD by Ca^{2+}-CaM is similar to that of two other plant Ca^{2+}-CaM–stimulated enzymes: radish plasma membrane Ca^{2+}-ATPase (Rasi-Caldogno et al., 1993) and pea nucleoside triphosphatase (Chen et al., 1987) activities were stimulated two- and threefold over the control by Ca^{2+}-CaM, respectively.

It is well known that the pH optimum for GAD in vitro is lower than the physiological pH (Bown and Shelp, 1989) and that many metabolic enzymes are sensitive to pH. Interestingly, increases in Ca^{2+} levels have been correlated with decreases in cytoplasmic pH (Felle, 1988). Thus, it is reasonable to propose that the Ca^{2+}-dependent CaM-induced activation of GAD may be influenced directly by other physiological processes acting in concert, such as a shift in cytosolic pH or stress-induced changes in substrate levels (Crawford et al., 1994). Whether the coupled stimulation of GAD by Ca^{2+}-CaM and lowered cytoplasmic pH completely accounts for the transiently increased accumulation of GABA during plant stress remains to be determined. In addition, it was recently shown that incubation of wheat roots in exogenous abscisic acid (ABA) led to a stimulation of in vivo GAD activity and an accumulation of GABA (Reggiani et al., 1993). Because ABA has been shown to raise intracellular Ca^{2+} levels (Gilroy et al., 1991), it is reasonable to suggest that ABA influences GAD activity in vivo through a Ca^{2+}-CaM–mediated process. To address these questions, further research on the association of Ca^{2+}, pH, and ABA with respect to GAD kinetics in vivo and in vitro is required.

GAD activity has been measured in leaves (Wallace et al., 1984), petals (Baum et al., 1993), mesophyll cells (Snedden et al., 1992), and cotyledons (Smith and Waygood, 1961; Tuin and Shelp, 1994) of various species. In contrast, a CaM-BP of ~62 kD was not detected in leaves and cotyledons of fava bean by the ^{125}I-CaM overlay assay (Ling and Assmann, 1992). A number of possibilities may account for the discrepancy between the lack of a 62-kD CaM binding protein in leaves and documented GAD activity: alternate isoforms of GAD may exist within plants (Inatomi and Slaughter, 1975; W.A. Snedden and B.J. Shelp, unpublished data), only some of which may be CaM-regulated; endogenous CaM may remain bound to GAD through the extraction and assay; the action of post-translational modifications alluded to below may alter the CaM binding property of GAD or the molecular mass of GAD; and

cofactors or conditions necessary for CaM binding to leaf GAD may be missing from the in vitro assay. In mammalian systems, multiple isozymes of GAD are known, with some forms exhibiting Ca^{2+}-dependent electrostatic membrane-binding activity (reviewed in Erlander and Tobin, 1991). One form of brain GAD has been reported to associate with presynaptic vesicles in a Ca^{2+}-dependent manner (Nathan et al., 1994). In distinction to plant GAD, mammalian GAD activity appears to be CaM independent (Gold, 1983).

Post-translational modifications may account for changes in the apparent size of the soluble CaM-BP between different tissues. Mobility retardation of an unidentified CaM-BP from 100 to 110 kD in PC-12 rat adrenal pheochromocytoma cells treated with nerve growth factor was the result of rapid and sustained protein phosphorylation (Brady and Palfrey, 1993). A model of GAD activation based on the phosphorylation state in brain has been proposed (Nathan et al., 1994). In addition to phosphorylation, post-translational protein modifications, such as ubiquitination (Jennissen and Laub, 1988; Ziengenhagen and Jennison, 1990) and limited proteolysis, may influence the mobility of proteins (Murtaugh et al., 1986; Jablonsky et al., 1991). Whether or not GAD undergoes such modifications remains to be determined.

The distribution of GAD in roots parallels that of methylated CaM, which is highest in differentiated roots and lowest in the first centimeter section of root tips (Oh and Roberts, 1990). Because the methylation state of CaM affects the activation of certain CaM-dependent enzymes (Roberts et al., 1986), and the overproduction of unmethylated CaM may influence the axial development of transgenic plants (Roberts et al., 1992), the distribution of the 62-kD CaM-BP may be coordinately regulated with that of methylated CaM in roots.

An additional level of complexity in understanding the mechanism of CaM–GAD interaction is introduced by the presence of multiple CaM isoforms in plant systems. Six independent CaM cDNA isoforms (Ling et al., 1991; Perera and Zielinski, 1992; Gawienowski et al., 1993) have been characterized from Arabidopsis; the cDNAs encode four protein isoforms that vary in amino acid sequence by up to four amino acids. In Arabidopsis, cDNAs encoding CaM-like proteins (Braam and Davis, 1990; Ling and Zielinski, 1993) have also been isolated. The biochemical significance of the different CaM isoforms and CaM-like proteins is not currently known, and methods of separating and purifying these isoforms and proteins from plant sources have not yet been devised. Because the gene expression pattern of each CaM isoform varies between organs, it is likely that the isoforms are differentially accumulated in plants. In this study, CaM isolated from fava bean roots was used in activity assays of root GAD, but it remains to be determined to what extent CaM protein(s) from other tissues modulate GAD activity.

The finding of the soluble 64-kD CaM-BP in total nodule extracts raises the intriguing possibility that Ca^{2+}-CaM play an integral role in *Rhizobium*–legume endosymbiosis. It is well known that complex cellular signaling occurs between legumes and *Rhizobium*, including infection thread initiation, cortical cell proliferation, and nodule formation (Fischer and Long, 1992). Recently, Ca^{2+} signaling was implicated in nodule signal transduction by the discovery that nodulin-26 is a substrate of the Ca^{2+}-dependent protein kinase (Weaver et al., 1991). By anti-CaM immunoblotting (Jablonsky et al., 1991), approximately twofold higher levels of CaM on a total protein basis were found in nodule extracts than in root extracts (data not shown), which is consistent with the elevated levels of CaM typically found in plant meristems and actively dividing tissues (Roberts and Harmon, 1992). Correspondingly, GABA accumulates in root nodules, suggesting the presence of high GAD activity in nodules (Antoniw and Sprent, 1978). Although the cellular localization of nodule GAD, its regulation by CaM, and the role it plays in nodule physiology remain to be determined, the compelling idea that Ca^{2+} signaling is an intrinsic part of nodule function may be significant in future biochemical studies of plant–bacteria symbioses.

In conclusion, we determined the identity of a 62-kD CaM-BP from the root as GAD and demonstrated by activity assays that GAD is a bona fide Ca^{2+}-CaM–stimulated enzyme. Future studies detailing CaM and GAD isozyme activity, along with conditions promoting activation of GAD both in vitro and in vivo, will aid in understanding the role of Ca^{2+}-mediated signal transduction in plant responses to stress.

METHODS

Plant Material and Growth Conditions

Seeds of fava bean (*Vicia faba* cv Long Pod; Harris-Moran Seed Co., Modesto, CA, and W. Atlee Burpee & Co., Warminster, PA) were germinated in vermiculite in growth chambers under 10-hr-light, 14-hr-dark cycles with light/dark temperatures of 21/19°C. For experiments using seedlings, the seedlings were harvested after ~2 weeks and washed with deionized water prior to use. For protein purification, plants were grown to maximize root mass. Plants were germinated and grown in 8-inch pots containing perlite in the bottom two thirds and vermiculite in the remaining third. Plants were regularly watered with one-quarter strength Hoagland's solution four times per week for 4 to 6 weeks and then harvested. For root nodule production, fava bean seeds were inoculated with *Rhizobium leguminosarum* bv *viciae* 248 (gift of H. Roest, Leiden University, Leiden, The Netherlands) prior to planting. Plants were watered with nitrogen-free one-quarter strength Hoagland's solution as given above. Nodules were harvested from roots after 4 to 6 weeks of growth.

Protein Extraction

Plant material was ground into a fine powder under liquid N_2 and suspended in an ice-cold extraction buffer comprised of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/mL leupeptin, 1 μg/mL pepstatin, and 14 mM β-mercaptoethanol. (All reagents were purchased from Sigma unless otherwise stated.) The extract was centrifuged at 16,000*g* for 5 min, and the pellet was discarded. For some experiments, the supernatant was ultracentrifuged

in an Airfuge (Beckman Instruments, Palo Alto, CA) at 30 psi (150,000*g*) at 4°C for 1 hr to separate the soluble fraction from the microsomal membrane pellet. Protein concentrations were determined by using the Bio-Rad protein assay kit.

^{125}I-Calmodulin Overlay Assay

Overlays were performed as previously described (Ling and Assmann, 1992) with several modifications. Nonreducing SDS-PAGE loading buffer (60 mM Tris-HCl, pH 7.5, 2% [w/v] SDS, 10% glycerol, and 0.1% [w/v] bromophenol blue) was used to resuspend precipitated proteins prior to boiling and separation by SDS-PAGE. The absence of reducing agent did not affect the migration or detection of ^{125}I-calmodulin (CaM) binding proteins described in this study (data not shown). CaM content of the binding buffer used in the overlay procedure was reduced to 1 μCi ^{125}I-CaM/10 mL of buffer (~10 nM CaM). With this concentration of ^{125}I-CaM, no Ca^{2+}-independent CaM binding proteins (CaM-BPs) were detected in the presence of 1 mM EDTA (data not shown). As a result of the minor variability between assays of the CaM specific activity and of the transfer efficiency of proteins, duration of autoradiography for each blot was empirically determined, ranging from 7 to 10 days. Densitometric quantitation and comparison of radiographic signals were performed only on overlays of blots of samples run in the same gel.

Partial Purification and Sequence Determination of the 62-kD CaM-BP

Four hundred grams of 4- to 6-week-old roots were used as starting material for each protein preparation, and all purification steps were performed at 4°C. Crude homogenates were made by blending with extraction buffer containing 10 mg/mL insoluble polyvinylpolypyrrolidone. The homogenate was filtered through two layers of Miracloth (Calbiochem, San Diego, CA) and centrifuged at 100,000*g* for 1 hr. The soluble fraction was batch adsorbed onto 50 mL of DEAE-cellulose (DE-52) with constant stirring for 40 min. DEAE-cellulose was packed into a 1.5-cm-diameter column and washed with 5 volumes of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and eluted by a 400-mL 0 to 500 mM NaCl linear gradient in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA over a 16-hr period. Eluted proteins were collected in 5-mL fractions. ^{125}I-CaM overlay assays indicated that the 62-kD CaM-BP peak eluted from DEAE-cellulose between 100 and 230 mM NaCl. Fractions containing the CaM-BP were pooled, brought to 5 mM $CaCl_2$, and passed through a 5-mL CaM-agarose column at 1 mL min^{-1}. After washing with 5-column volumes of 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM $CaCl_2$, proteins were eluted with 50 mM Tris-HCl, pH 7.5, 5 mM EGTA at 1 mL min^{-1}. Electroblot and ^{125}I-CaM overlay assay following SDS-PAGE (Ling and Assmann, 1992) of the proteins in the final fraction revealed the presence of several CaM-BPs, including a dominant 62-kD CaM-BP corresponding to a protein band visualized by Coomassie Brilliant Blue R 250 staining of the polyvinylidene difluoride membrane (Immobilon-P PVDF membranes; Millipore, Bedford, MA). Excision of the band from PVDF membranes followed by determination of protein content by amino acid composition analysis indicated a purification of ~7000-fold and 1.4% yield (Table 1). Based on these data, 200 ng of 62-kD CaM-BP could easily be detected by using the ^{125}I-CaM overlay assay.

Six preparations were performed sequentially and accumulated, with the final protein fractions frozen under liquid N_2 and stored at −20°C. The final fractions were thawed to 4°C, combined, and precipitated with 5% trichloroacetic acid and then centrifuged at 16,000*g*. The resultant pellets were washed with 100% acetone, resuspended in nonreducing SDS-PAGE buffer and boiled for 1 min. Proteins were then separated by SDS-PAGE and electrotransferred to PVDF as previously described (Ling and Assmann, 1992). A band at 62 kD was visualized by staining with 0.1% (w/v) Ponceau S, 1% (v/v) acetic acid followed by destaining in water. The Ponceau S–stained band, which corresponded exactly with the location of a ^{125}I-CaM binding band in a side-by-side duplicate lane, was excised and submitted to the Harvard University Microchemistry Facility. Tryptic digestion of the protein, HPLC separation of proteolyzed fragments, and peptide sequence determination were performed.

CaM Purification

Clarified extracts were prepared from fava bean roots, as given above, and purified by DEAE column chromatography and phenyl-Sepharose column chromatography according to the protocol described by Ling and Assmann (1992). The final CaM fraction was desalted by G-25 column chromatography and stored at −20°C.

Glutamate Decarboxylase Assays

All procedures were performed at 4°C unless stated otherwise. One- to two-week-old seedlings were harvested, immediately frozen in liquid nitrogen, and stored at −70°C. Frozen root tissue was rinsed with deionized water and then ground with a mortar and pestle in 5 volumes of 100 mM bis-Tris-HCl buffer, pH 7.0, containing 5 mM EDTA, 0.5 mM pyridoxal-5′-phosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.5% (w/v) polyvinylpolypyrrolidone (insoluble). The homogenate was centrifuged at 23,000*g* for 20 min, and the pellet was discarded. The supernatant was brought to 30% (w/v) ammonium sulfate, stirred for 45 min, and then centrifuged as given above. The resultant supernatant was decanted, brought to 60% (w/v) ammonium sulfate, stirred for 45 min, and then centrifuged as described above. The final pellet was solubilized in extraction buffer and desalted by passage through a Sephadex G-25M PD-10 column (Pharmacia). The desalted sample was frozen in liquid nitrogen and stored at −80°C.

The thawed sample was passed through a 0.45-μm filter (Millipore) and loaded onto a Protein-Pak DEAE 8HR anion exchange column (Waters, Milford, MA) (10 mm × 100 mm) preequilibrated with 50 mM bis-Tris-HCl buffer, pH 7.0, containing 1 mM EDTA, 1 mM DTT, and 10% glycerol at a flow rate of 1.0 mL min^{-1}. Proteins were eluted using a 70-min linear gradient of 0 to 1 M NaCl in the column equilibration buffer as described above. Pyridoxal-5′-phosphate was included in the collection tubes at a final concentration of 0.1 mM. The fractions eluting in the region between 0.30 to 0.41 M NaCl were pooled and then desalted and concentrated using a Centriprep-30 concentrator (Amicon, Beverly, MA).

The concentrated sample was assayed for GAD activity as L-1-^{14}C-glutamate–dependent $^{14}CO_2$ production (Snedden et al., 1992). Samples (100 μL) were preincubated in a shaking water bath at 30°C for 30 min in 34-mL sealed serum vials (Wheaton, Millville, NJ) containing 100 mM bis-Tris-HCl buffer, pH 7.0, 1 mM DTT, 0.5 mM pyridoxal-5′-phosphate, and 10% glycerol in a final volume of 1.95 mL. Purified fava bean root CaM and Ca^{2+} (as $CaCl_2$) were added during preincubation as required to final concentrations of 100 nM and 100

μM, respectively. EGTA or EDTA was not included because these chelators were found to competitively inhibit glutamate decarboxylase (GAD) activity in this assay system (W.A. Snedden and B.J. Shelp, unpublished data). After 30 min, a CO_2 trap containing 0.5 mL of 0.1 N NaOH (prepared fresh daily) was added to the serum vial, and the reaction was initiated by the addition of L-glutamate to a final concentration of 2 mM (5 μCi/mmol). Vials were incubated for an additional 30 min. The reaction was terminated by the injection of 0.1 mL of 18 N H_2SO_4 to the reaction medium. Vials were left at 4°C overnight before the carbon 14 content of the CO_2 trap was determined by liquid scintillation spectrometry. No activity was detected in assays in the absence of root extract. For protein determination, a 50-μL sample was precipitated with 12% trichloroacetic acid (w/v) and the resolubilized pellet assayed by the method of Bradford (1976).

ACKNOWLEDGMENTS

We thank Janet Sherwood for the maintenance of fava bean seedlings. This work was supported by National Aeronautics and Space Administration Grant No. NAGW-70 to V.L., Natural Sciences and Engineering Research Council (Canada) Grant No. U0343 to B.J.S., and United States Department of Agriculture Grant No. 92-37100-7537 to S.M.A.

Received March 4, 1994; accepted June 23, 1994.

REFERENCES

Antoniw, L.D., and Sprent, J.I. (1978). Primary metabolites of *Phaseolus vulgaris* nodules. Phytochemistry **17,** 675–678.

Baum, G., Chen, Y., Arazi, T., Takatsuji, H., and Fromm, H. (1993). A plant glutamate decarboxylase containing a calmodulin binding domain. J. Biol. Chem. **268,** 19610–19617.

Bethke, P.C., and Jones, R.L. (1994). Ca^{2+}-calmodulin modulates ion channel activity in storage protein vacuoles of barley aleurone cells. Plant Cell **6,** 277–285.

Bown, A.W., and Shelp, B.J. (1989). The metabolism and physiological roles of 4-aminobutyric acid. Biochem. (Life Sci. Adv.) **8,** 21–25.

Braam, J., and Davis, R.W. (1990). Rain-, wind- and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis.* Cell **60,** 357–364.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Brady, M.J., and Palfrey, H.C. (1993). Rapid and sustained phosphorylation of a calmodulin-binding protein ($CaM\text{-}BP_{100}$) in NGF-treated PC12 cells. J. Biol. Chem. **268,** 17951–17958.

Chen, Y., Datta, N., and Roux, S.J. (1987). Purification and partial characterization of a calmodulin-stimulated nucleoside triphosphatase from pea nuclei. J. Biol. Chem. **262,** 10689–10694.

Crawford, L.A., Bown A.W., Breitkreuz, K.E., and Guinel, F.C. (1994). The synthesis of γ-aminobutyric acid in response to treatments reducing cytosolic pH. Plant Physiol. **104,** 865–871.

Erlander, M.G., and Tobin, A.J. (1991). The structural and functional heterogeneity of glutamatic acid decarboxylase: A review. Neurochem. Res. **16,** 215–226.

Felle, H. (1988). Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L. Interaction of calcium and pH? Planta **176,** 248–255.

Fischer, R.F., and Long, S.R. (1992). *Rhizobium*-plant signal exchange. Nature **357,** 655–660.

Gawienowski, M.C., Szymanski, D., Perera, I.Y., and Zielinski, R.E. (1993). Calmodulin isoforms in *Arabidopsis* encoded by multiple divergent mRNAs. Plant Mol. Biol. **2,** 215–225.

Gilroy, S., Fricker, M.D., Read, N.D., and Trewavas, A.J. (1991). Role of calcium in signal transduction of *Commelina* guard cells. Plant Cell **3,** 333–344.

Gold, B.I. (1983). Further studies on the role of calcium in the regulation of glutamate decarboxylase activity in brain slices. Neurochem. Res. **8,** 185–191.

Guinn, G., and Brinberhoff, L.A. (1970). Effect of root aeration on amino acid levels in cotton plants. Crop. Sci. **10,** 175–178.

Inatomi, K., and Slaughter, J.C. (1975). Glutamate decarboxylase from barley embryo and roots: General properties and the occurrence of three enzymic forms. Biochem. J. **147,** 479–484.

Jablonsky, P.P., Grolig, F., Perkin, J.L., and Williamson, R.E. (1991). Properties of monoclonal antibodies to plant calmodulin. Plant Sci. **76,** 175–184.

Jackson, F.R. (1990). Prokaryotic and eukaryotic pyridoxal-dependent decarboxylases are homologous. J. Mol. Evol. **31,** 325–329.

Jennissen, H.P., and Laub, M. (1988). Ubiquitin-calmodulin conjugating activity from cardiac muscle. Biol. Chem. Hoppe-Seyler **369,** 1325–1330.

Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effect of touch and cold shock and elicitors on cytoplasmic calcium. Nature **352,** 524–526.

Kobayashi, Y., Kaufman, D.L., and Tobin, A. (1987). Glutamic acid decarboxylase cDNA: Nucleotide sequence encoding an enzymatically active fusion protein. J. Neurosci. **7,** 2768–2772.

Kulkarni, L., and Sohonie, K. (1956). Glutamic acid decarboxylase in legumes. Nature **178,** 925.

Leonard, R.T., Nagahashi, G., and Thompson, W.W. (1975). Effect of lanthanum on ion absorption in maize roots. Plant Physiol. **55,** 542–546.

Ling, V., and Assmann, S.M. (1992). Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba* L. Plant Physiol. **100,** 970–978.

Ling, V., and Zielinski, R.E. (1993). Isolation of an *Arabidopsis* cDNA sequence encoding a 22 kDa calcium-binding protein (CaBP-22) related to calmodulin. Plant Mol. Biol. **2,** 207–214.

Ling, V., Perera, I.Y., and Zielinski, R.E. (1991). Primary structures of *Arabidopsis* calmodulin isoforms deduced from the sequences of cDNA clones. Plant Physiol. **96,** 1196–1202.

Maras, B., Sweeney, T., Barra, D., Bossa, F., and John, R.A. (1992). The amino acid sequence of glutamate decarboxylase from *Escherichia coli*: Evolutionary relationship between mammalian and bacterial enzymes. Eur. J. Biochem. **204,** 93–98.

Murtaugh, T.J., Wright, L.S., and Siegel, F.L. (1986). Posttranslational modification of calmodulin in rat brain and pituitary. J. Neurochem. **47,** 164–172.

Nathan, B., Hsu, C.-C., Bao, J., Wu, R., and Wu, J.-Y. (1994). Purification and characterization of a novel form of brain L-glutamate decarboxylase. J. Biol. Chem **269,** 7249–7254.

Neuhaus, G., Bowler, C., Kern, R., and Chua, N.-H. (1993). Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell **73,** 937–952.

Oh, S.-H., and Roberts, D.M. (1990). Analysis of the state of post-translational calmodulin methylation in developing pea plants. Plant Physiol. **93,** 880–887.

Perera, I.Y., and Zielinski, R.E. (1992). Structure and expression of the *Arabidopsis* CaM-3 calmodulin gene. Plant Mol. Biol. **19,** 649–664.

Rasi-Coldogno, F., Carnelli, A., and De Michelis, M.I. (1993). Controlled proteolysis activates the plasma membrane Ca^{2+} pump of higher plants. Plant Physiol. **103,** 385–390.

Reggiani, R., Nicoletta, A., Mattana, M., and Bertani A. (1993). ABA induces 4-aminobutyrate accumulation in wheat seedlings. Phytochemistry **34,** 605–609.

Roberts, D.M., and Harmon, A.C. (1992). Calcium-modulated proteins: Targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43,** 375–414.

Roberts, D.M., Rowe, P.M., Siegel, F.L., Lukas, T.J., and Watterson, D.M. (1986). Trimethyllysine and protein function: Effect of methylation and mutagenesis of lysine 115 of calmodulin on NAD kinase activation. J. Biol. Chem. **261,** 1491–1494.

Roberts, D.M., Besl, L., Oh, S.-H., Masterson, R.V., Schell, J., and Stacey, G. (1992). Expression of a calmodulin methylation mutant affects the growth and development of transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **89,** 8394–8398.

Satya Narayan, V., and Nair, P.M. (1990). Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. Phytochemistry **29,** 367–375.

Smith, J.E., and Waygood, E.R. (1961). Resolution of the apoenzyme and coenzyme of L-glutamic acid decarboxylase from sunflower cotyledons. Can. J. Biochem. Physiol. **39,** 1055.

Snedden, W.A., Chung, I., Pauls, R.H., and Bown, A.W. (1992). Proton/L-glutamate symport and the regulation of intracellular pH in isolated mesophyll cells. Plant Physiol. **99,** 665–671.

Stinemetz, C.L., Kuzmanoff, K.M., Evans, M.L., and Jarrett, H.W. (1987). Correlation between calmodulin activity and gravitropic sensitivity in primary roots of maize. Plant Physiol. **84,** 1337–1342.

Tuin, L.G., and Shelp, B.J. (1994). *In situ* [^{14}C]glutamate metabolism by developing soybean cotyledons. I. Metabolic routes. J. Plant Physiol. **143,** 1–7.

Wallace, W., Secor, J., and Schrader, L.E. (1984). Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol. **75,** 170–175.

Weaver, C.D., Crombie, B., Stacey, G., and Roberts, D.M. (1991). Calcium-dependent phosphorylation of symbiosome membrane proteins from nitrogen-fixing soybean nodules. Plant Physiol. **95,** 222–227.

Ziengenhagen, R., and Jennissen, H.P. (1990). Plant and fungus calmodulins are polyubiquitinated at a single site in a Ca^{2+}-dependent manner. FEBS Lett. **273,** 253–256.